A MULTIDISCIPLINARY ASSESSMENT OF LOW DOSES OF HYDROGEN SULFIDE

A PROJECT FUNDED BY THE
OCCUPATIONAL HEALTH AND SAFETY
HERITAGE GRANT PROGRAM

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OF

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NOTE FROM THE

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PROJECT FINAL REPORT

to

ALBERTA OCCUPATIONAL HEALTH AND SAFETY

HERITAGE GRANT PROGRAM

A Multidisciplinary Assessment of Low Doses of Hydrogen Sulphide

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EXECUTIVE SUMMARY

The overall objective of the study was to determine whether exposure to low levels of hydrogen sulphide (H_2S) alters the structure and/or function of various tissues of the mature and immature mammalian system using the rat as the test animal.

The project was designed as a multidisciplinary project in order to determine quantitatively whether low concentrations of H_2S produced any adverse effects on a variety of parameters including behavioral, morphological, neurological, biochemical and genetic levels. We focused on the developing system because of the potentially greater susceptibility of this age group. The majority of the objectives were completed, and the final analyses revealed that low levels of H_2S can produce toxic effects on the developing organism.

Many of the results exhibit and emphasize the "subtle" nature of the toxicity of low levels of hydrogen sulphide. Threshold effects may not have been identified because of the concentration range examined, and may be considerably lower than previous estimates. The extensive screening program undertaken in this laboratory during the past three years generated a large volume of data on many diverse parameters.The data that proved to be not significant are the most difficult to justify in a progress report, however it was absolutely necessary that these data be obtained in a well controlled study, and now the results can be documented with confidence. Research involving inhalation exposures and multidisciplinary assessments of developing organisms are very tedious, prolonged, labour intensive and relatively slow to completion.In general, the results of the study revealed that development of the rat fetus and neonate (to day 21 postnatal) appears to be normal with no evidence of any overt teratological effects. Routine parameters indicative of normal development were not significantly different in the exposed group (20, 50 and 75 ppm H_2S) from the control group.There did not appear to be any significant differences in hematological parameters such as hematocrit, nor

pathological or histopathological parameters between exposed or control groups.

A number of significant effects, however, did occur. Prolongation of parturition (dystocia) was observed in approximately 18% of the 75 ppm exposed group.In vitro studies provided evidence that this phenomenon may be a result of H_2S-induced disruption of disulphide bonds in the oxytocin receptor.Serum glucose levels of exposed dams were elevated accompanied by a dose-dependent decrease in serum triglycerides and elevation of serum cholesterol. Further studies are required to provide explanations. Overall we did not find any major alterations in the biochemical profiles of either the dams or the pups exposed to low levels of H_2S, however there were several indications that changes in some metabolites and brain enzymatic activity were transitorily altered. Initial genotoxicity studies revealed that the frequency of micronuclei in liver cells of exposed animals (75 ppm H_2S) was lower than control tissues suggesting that mutagenesis may occur at these levels of H_2S. Further studies are required to evaluate significance of this effect. Preliminary studies using fetal lung fibroblasts in culture also demonstrated that growth was inhibited following a single exposure to Na_2S.

The dominant effects of H_2S appear to occur in the developing central nervous system. We have documented that H_2S at the concentrations studied alters the amino acid levels in the developing rat cerebrum and cerebellum during the critical phase of development; the population densities of Purkinje and granule cells in the cerebellum are changed and the growth characteristics of the Purkinje cells are significantly less random that controls. Studies using in vitro neuronal models also revealed that low concentrations of H_2S can produce a time and concentration -dependent alteration of discharge activity without significant change in conduction velocity along the axon.

During the initial phase of the project we designed and fabricated an ideal exposure system for conducting whole animal experiments that is

now being developed for commercial distribution by a company in Calgary. The techniques utilized in this study were constantly refined and resulted in routine procedures that are consistent, reliable and suitable for future studies to assess the toxicity of low levels of toxicants such as H_2S.

As expected, the research generated many new problems, and provided the foundation for well-designed future research.The project still exists in the "beginning stages", however continued research is dependent on sufficient funding. Until a solution to this problem is found, many of the questions concerning hydrogen sulphide toxicity will remain unanswered, and the findings of the present study will not be utilized to the fullest extent. Since an environmental toxicant such as H_2S does not exist in isolation, it is realistic to conduct future studies using combinations of agents, and chronic exposures for longer periods of time at lower concentrations.

OBJECTIVES OF THE STUDY

Overall objective: to determine whether exposure to low levels of hydrogen sulphide (H_2S) alters the structure and/or function of various tissues of the mature and immature mammalian system using the rat as the test animal.

The project began in July, 1986. **The objectives of the first year** of study were to (1) complete the installation of the equipment such as the inhalation exposure chamber apparatus, (2) identify and train technical personnel, (3) test equipment required for the exposures and analyses, (4) establish the procedures required for monitoring the animals, collecting samples and tissue analysis, and (5) initiate preliminary control and H_2S experiments on two populations.

The objectives for the second year were to assess the effects of low dose exposure of H_2S (<50 ppm) on postnatal development using established procedures on:

(a) physical development, e.g. weight gain, pinna detachment, incisor eruption, and eye opening

(b) behavioral and neuromuscular tasks such as righting reflex, negative geotaxis and walking gait

(c) morphometric analyses of the brain applying conventional histologic techniques,e.g. gross measurements of cortical and medullary thickness of cerebellum and hippocampus

(d) neurochemical parameters, e.g.levels of chemical neurotransmitters

(e) biochemical development in liver and brain,e.g. key marker enzymes such as cytochrome oxidase, alkaline phosphatase, liver glycogen synthetase, and liver cytochrome P450

(f) insulin and epidermal growth factor receptors in liver and brain

(g) genotoxicity of brain and liver using micronuclei assay procedures

To evaluate the toxicity of low H_2S exposure on the adult system using the same procedures outlined above.

To study the effects of Na_2S (the non-volatile salt of H_2S) on in vitro tissue and culture preparations in order to establish procedures for screening and to determine mechanisms of action

To conduct routine morphometric analysis on most of the of the brain tissue of the exposed and control groups. These experiments were to utilize histological preparations and analysis was to be performed using developed computer-assisted methods.

To initiate neurochemical studies on brain tissue and compare the results with the morphological results.

To obtain control data for the genotoxicity studies and compare these with exposed tissue at 75 ppm H_2S.

To complete initial studies utilizing the in vitro neuronal preparation.

To begin experiments using the hepatocyte cultures in order to assess the biochemical effects of H_2S on another in vitro system.

To develop the analytical procedures for the determination of sulfhemaglobin and to evaluate whether this technique would be useful as a clinical tool in the assessment of human exposures.

The major goal for the third year was to complete the objectives stated in the original proposal.

To complete the exposure studies at 20, 50 and 75 ppm H_2S with matched controls and to attempt to generate dose response curves and determine threshold levels for the various parameters measured.

To incorporate a modified exposure protocol which included the major portion of the gestational period and to continue until day 21 postnatal.

To expand and complement the ongoing genotoxicity studies by using established in vitro culture techniques in order to demonstrate a correlation between mitotic index and micronuclei frequency.

To investigate the role of prostaglandins and oxytocin in relation to the observed dystocia on exposure to H_2S during studies conducted in Phase 2.

To extend the number of biochemical assays using the acquired Multistat III Autoanalyzer and thus provide a larger and broader profile for comparison.

To complete the in vitro studies using the isolated neuronal preparation and the fibroblast cultures, and to evaluate whether these in vitro systems would be useful as screening methods, models for elucidation of mechanisms of action and/or antidotal therapy.

To document the pathological and histopathological effects of H_2S in both exposed and matched controls in collaboration with Dr. P.N. Nation, Head of the Pathology Branch, Aniaml Health Division, Alberta Agriculture, Edmonton.

MATERIALS DEVELOPED

The essential equipment required for inhalation studies is a suitable exposure chamber apparatus. We were able to design and fabricate a suitable system that was ideal for conducting whole animal exposures. Details of the apparatus have been described in previous Progress Reports. Briefly the chambers were made from two acrylic domes (Chinook Plastics Alta) of approximately 90 liters in volume. The chamber design allows continuous observation during exposure and ensures uniform distribution of gas mixtures. The animals are kept in a circular-shaped wire cage within the chamber during the exposure. The cages are separated into six compartments. Two adults or one dam plus litter can be housed per compartment during the exposure period. The total animal volume does not exceed five percent of the total chamber volume and the chambers each have an inlet orifice plate sized and calibrated for 20 air changes per hour which permits adequate thermal regulation and keeps animal surface effects from affecting the chamber concentration as well as minimizing odors from animal excrement.The pressure taps on each side of the orifice plate are connected to a magnehelic gauge (Barber Engineering) which has been calibrated for flow in litres per minutes. The slight negative pressure maintained in the chamber is measured with a water manometer (Fisher Scientific). Temperature in the chamber is continuously monitored via a thermistor probe and digital readout. The air supply to the chamber is room air drawn through the chamber by a vacuum blower. The clean air is combined with H_2S (2000 ppm in nitrogen) supplied by Matheson Gas (Edmonton, Alta.). The use of a 2000 ppm H_2S supply results in a dilution factor which will permit atmospheric conditions to remain normal (i.e. oxygen concentration maintained at 20-21 percent). The mixed air/H_2S then passes over the orifice plate, into the chamber and through a diffuser. The air passes through multiple drainage slits to a dual exhaust pipe at the bottom of the chamber. All exhaust air passes through a sodium hydroxide water trap to remove H_2S before being vented into an exhaust ventilation duct.

The concentration of H_2S within the chamber is continuously monitored near the breathing zone of the animals by using a pre-calibrated digital GFG model GMA100 H_2S monitoring system (Levitt Safety, Calgary, Alta.). Temperature and H_2S concentrations are recorded on a strip chart recorder or Data Logger.

Although we were encouraged by many to publish the design and characteristics of our exposure system , we were advised to develop a system that could be commercially viable. With the assistance of the University of Calgary Technology Transfer Office , we were successful in being awarded a Technology Transfer Grant from the Alberta Heritage Foundation for Medical Research. This grant provided the funds to design a modular environmental chamber suitable for a variety of research applications. A Calgary based company has stated an intent to manufacture this chamber, and a prototype should be available by the middle of 1990.

In addition to the equipment, a number of methods, procedures and techniques were developed or refined during the course of the project. We established methods which proved to be reproducible and realiable,which can accurately assess the toxicity of low levels of toxicants such as H_2S.In addition, our approaches using basic research techniques may provide the basis for a number of procedures that could be useful in pre-clinical (human) studies.

The research program on H_2S provided the stimulus to establish a Division of Toxicology at the University of Calgary and a Toxicology Interest Group under the leadership of Dr. Sheldon H. Roth. The Division has the potential for growth into a recognized academic centre for toxicology research. At the present time the Interest Group consists of more than 40 individuals from the University, Government, Industry and Hospitals. It is meeting on a regular schedule and provides the basis for interactive discussions and collaborative projects.

As a result of the recent interest and funding of H_2S research, a group of individuals from the Universities of Calgary and Alberta and the Alberta Environmental Research Centre have formed a Sulphide Research Group , chaired by Dr. S. Roth. This group organized the first International Conference on Hydrogen Sulphide Toxicity which was recently held in Banff, June 1989. The proceedings of this Conference are currently in press and will soon be available as a monograph.

RESULTS OF THE STUDY

As reported in the previous Progress Reports submitted to the Occupational Health and Safety Heriatge Grant Program, many of the results emphasize the "subtle" nature of the toxicity of low levels of H_2S. We have observed that the dominant effects of H_2S appear to occur in the developing central nervous system. This represents the major direction of the recent studies in this laboratory. We have attempted to establish dose-response relationships for most of the studies; however, there is good evidence that some of the toxic effects may be enhanced at lower concentrations of H_2S than examined in the present study. Threshold effects may not have been identified because of the concentration range examined, and may, in fact, be considerably lower than previous published estimates.

The extensive screening program undertaken in this laboratory generated a large volume of data on many diverse parameters which indicated no significant effects of H_2S, i.e. negative results. These are perhaps the most difficult to justify in a progress report; but the results can now be documented with confidence. It can be argued that the numbers of test subjects may not have been large enough to identify significant "subtle' effects; but it is more likely that the techniques lack the precision required.

In an attempt to present the large volume and variety of data in a concise manner, brief descriptions are provided below with attached appendices and a list of publications.

The project still exists in the "beginning stages" but provides the foundation for continued research in this area of toxicology. It must be pointed out that the decision to focus on the neurotoxicological aspects of more recent and future studies was primarily based on the positive results and to some degree the expertise of the senior investigators. This decision should not convey the impression that we do not recognize the necessity of continued research in other fields. As a cost-effective measure in this area of research, a group such as

the Sulphide Research Network could collaborate on future studies to enhance the multidisciplinary approach, and to avoid costly duplication.

PROGRESS DETAILS

Exposure Chamber.
During the initial phase of this project we designed and fabricated an ideal inhalation exposure chamber that was very successful for conducting the whole animal exposures to low concentrations of H_2S. A modified version of this chamber will be developed by a company in Calgary suitable for a wide variety of research purposes.

Routine procedures for inhalation experiments.
Our techniques during the course of study were constantly improved, and we were able to establish routine procedures that resulted in consistent and reliable data. Initial studies were focused on obtaining baseline (control) data on various parameters for both maternal and fetal subjects. Comparison with published results were made if possible, however the majority of the data generated in the present study have not been previously reported.

Physical growth and development during subchronic exposure to low levels of H_2S.
Development of the rat fetus and neonate (to day 21 pn) appears to be normal with no evidence of any overt teratological effects. We have concluded that such parameters indicative of development (body and organ weight, gestation length, litter viability, litter size, male/female ratio, pinna detachment, hair development, incisor eruption, eyelid opening and surface righting) were <u>not</u> significantly different in the exposed group (20, 50 and 75 ppm H_2S) from the control group.We did not observe any possible complications of malnutrition due to water and food consumption, therefore the biochemical and morphological effects we observed were attributed to the effects of H_2S per se.

There was an initial decrease in maternal weight gain for all groups between days 5 to 9 of gestation. The 75 ppm group exhibited a greater decrease than either the 50 ppm or control. From day 9 to 21 the

weight gain was similar for the 50 and 75 ppm groups but differed from control. Food and water intake for all groups were similar.

H_2S-induced prolongation of parturition.

Significant prolongation of parturition (dystocia) was observed in a portion of the exposed groups.We reported that approximately 18% of the 75 ppm exposed group of pregnant rats exhibited dystocia; an average 120 minutes compared to 80 minutes for control. This event was not associated with a signification decrease in live birth rates or fetal toxicity. In collaboration with Dr. G. Moore, Department of Biochemistry, in vitro studies utilizing isolated uterine (smooth) muscle strips suspended in tissue baths have been carried out to identify possible explanations for the dystocia. It has been demonstrated that 30 μM Na_2S (volatile salt of H_2S) causes a reduction of the oxytocin-stimulated uterine muscle contraction. The oxytocin stimulated responses returned to normal upon removal of Na_2S solutions with fresh control wash. The results provide evidence that H_2S directly inhibits uterine contractions perhaps by disrupting the disulphide bridges of the oxytocin receptor. Previous pilot studies in this laboratory demonstrated that H_2S also decreased insulin and epidermal growth factor receptor numbers in rat hepatocytes.

Maternal Serum Glucose Levels

It was observed that the serum glucose levels of the dams exposed to H_2S were elevated significantly compared to controls. The increase in serum glucose was accompanied by a dose-dependent decrease in serum triglycerides and elevation of serum cholesterol. Significant changes in glucose levels were not observed in the developing pups; however, similar alterations of serum triglycerides and cholesterol were recorded in the developing pups as in the dams. The changes were suggestive of a trend which may be more dramatic with continued exposure and maturation (90 day exposure).

Biochemical Profiles

Overall we did not identify any major alterations in the biochemical profiles of either the dams or the pups exposed to low levels of H_2S. However, there were several indications that significant changes of

some metabolites and brain enzymatic activity were transitorily altered. At day 21 postnatal liver cholesterol and blood glucose levels were both significantly increased in the treated dams. In addition, at 75 ppm there was a significant decrease in brain cytochrome oxidase and significant increase in brain alkaline phosphatase activity in the treated dams. Transitory alterations in brain and liver cytochrome oxidase and alkaline phosphatase were noted at various times and doses in the pups. It is interesting to note that compared to the dams, the pups were surprisingly resistent to the treatment in terms of the parameters examined. Whatever mechanisms are involved during the growth phase that impart the apparent protection are certainly worthy of future investigation.

Hematology

We had anticipated an increase in hematocrit levels due to the stress of H_2S exposure. Hemoglobin levels were detrmined in both exposed and controlled dams to determine whether H_2S (50 or 75 ppm) altered red blood cell chemistry. We observed no significant effects on hematocrit (hemoglobin content) on exposure to H_2S.

Determination of Sulfhemoglobin (SHb)

The objective of this study was to determine whether low levels of sulfhemoglobin could be detected in the blood using simple absorbance spectrophotometry, and whether the amount of sulfhemoglobin could be directly related to H_2S exposure. Isolated erythrocytes from rats were exposed to various solutions of Na_2S (volatile salt of H_2S). It was possible to produce levels of SHb as high as 80% by exposing cells to extremely high concentrations of Na_2S (0.01 M).It was concluded, however,that absorbance scanning was not a sufficiently reliable method for determining SHb formation. The formation of SHb is an irreversible process. Exposure of cells to concentrations of 2.5mM and lower produces some sulphur species which have not been identified and interfer with the absorbance. Another method such as multi-component analysis could be considered in future studies.

Pathological and Histopathological Findings of Control and Exposed Animals

Up to the present time, the results have not provided any definitive or significant differences between the control and treated; however, there were a number of observations which are suggestive of H_2S-induced effects.

All animals that are sacrificed or die unexpectedly were examined in this laboratory for gross pathology. Any tissues that showed any abnormalities were prepared using conventional techniques and shipped to Dr. P.N. Nation, Head of the Pathology Branch, Animal Health Division, Alberta Agriculture, Edmonton. Dr. Nation was formerly a member of this laboratory and agreed to collaborate on this portion of the project. In addition to providing a report of the pathology of each specimen, sections were selected for histopathology. In females exposed to high concentrations of H_2S gas, reactive changes have been observed in the pulmonary alveoli. These changes have been well described in previous studies and are an expected finding. By far, the majority of histopathologic findings have been of changes that occurred as a result of the method of sacrifice of the rats. In most cases, this has been due to acute inhalation of blood at the time of death. Occasional animals have been found to be suffering from disease processes that are not of aany clinical significance and are unrelated to H_2S exposure. A summary of the data generated is listed:

General pathological and histopathological findings

Pups: focal areas in the liver of bacterial origin
heart defect
hemorrhage in anterior lobes of lung
atelectatic lungs
hemorrhage in liver
cystic kidney and ureter
mild hyperplasia of airway epithelium
bronchial lymphoid tissue indicative of antigenic stimulation
congestive heart failure
enlarged heart
eye and nose irritation at 75 ppm

Dams: interstitial pneumonia
inflammatory reaction of alveolar walls
low level of blood loss into lung tissue
hemorrhage in mammary tissue
atelectatic lung
necrotic hepatitic tissue
mammary gland adenoma - benign
increased number of stillborn births at 50 and 75 ppm
(not conclusive)

Genotoxicity Studies

The original objective was to assess the effects of low dose exposure of H_2S using the micronucleus assay as an indication of mutagen sensitivity. This portion of the study was not completed. Control data from tissues of newborn rat revealed that untreated samples contained relatively few micronuclei (approximately 2%). These values were obtained from scoring 1250 cells when the coefficient of variation was optimal. The frequency of micronuclei in the exposed (75 ppm H_2S) liver tissue of newborn rat was found to be 1.4 ± 0.5% compared to control (untreated) values of 2.3 ± 0.6%, a difference of 64% of control. In brain tissue, the average micronuclei frequency of the treated newborn rat was also reduced to 2.0 ± 0.6% from control levels of 2.5 ± 0.7% (25% difference).

The assay is very labour intensive and requires considerable training time. Without the availability of automated procedures, it was recommended by one of the investigators (Dr. D. Hoar) that this portion of the study be postponed on the basis that it was not cost-effective with the present technology. It had been proposed to continue the study using cultured cells, however, this has not progressed as well as planned, and did not fit within the time frame of the project. During the project, Dr. Hoar was re-located from the Health Science Centre to the Alberta Children's Hospital, Calgary which also contributed to the disruption of the study.

Effects of H_2S on Developing Central Nervous System

Amino Acid Analysis

Putative amino acid neurotransmitter levels in the rat cerebrum and cerebellum were determined to evaluate the effects of exposure to hydrogen sulfide during perinatal development. The levels of aspartate, GABA, glutamate, glycine and taurine were quantitated using high performance liquid chromatography. With the exception of glycine, all of the amino acids were affected by the treatment. On day 21 postnatal, which was the last day of the exposure, aspartate, glutamate and GABA in the cerebrum and aspartate and GABA in the cerebellum were significantly depressed. The observed alterations in the amino acid levels during this critical phase of development may have chronically affected the activity of the neurotransmitters, their receptor sensitivity or their individual target areas. The consequence of one or a combination of such alterations may lead to behavioral and structural abnormalities.

Morphometric Analysis - Neuronal Populations - Cerebellum

The population densities of Purkinje and granule cells in the cerebellum were quantitated on days 7 and 14 postnatal (75 ppm exposure). The treatment produced a significant increase in the density of the treated Purkinje cells and a significant decrease in the ratio of granule cells to Purkinje cells.

Vertex Analysis - Purkinje Cells - Cerebellum

Vertex analysis of the treated Purkinje cells at day 21 postnatal revealed several significant alterations in both the dendritic architecture and the overall growth process. the treated cells extended their dendritic processes significantly further before branching than did the control cell. In addition, the treated cells produced a significantly greater number of branches within the middle of the dendritic field. In terms of growth characteristics, a comparison of branch type ratios demonstrated that the treated cells were significantly less random in their growth than comparable control cells.

H_2S-Induced Alteration of Neuronal Activity

Using the stretch receptor neuron of the crayfish as an isolated neuronal preparation, we have demonstrated that low concentrations of H_2S produce a time and concentration-dependent alteration of discharge activity without significant change in conduction velocity along the axon.

Effects of H_2S on Human Lung Fibroblast Growth Kinetics

The objective of this study was to determine the effect of Na_2S on the growth kinetics of cells grown in culture. The studies demonstrated that the growth of fetal human lung fibroblasts (WI-38) was inhibited following a single exposure to 250 uM Na_2S for 24 hours. Protein content was suppressed by 12% and cellular DNA accumulation was 30% less than control.

PROJECT PERSONNEL

Dr. Sheldon H. Roth, Professor, Principle Investigator
Dr. Richard S. Hannah, Associate Professor, Co-investigator
Dr. Lawrence J. Hayden, Assistant Professor, Co-investigator
Dr. Helen Goeden, AHFMR Post-Doctoral Fellow
Ms. H. Mathison: Chief technician primarily responsible for exposures, isolated neuronal studies and data acquisition.
Mr. R. Bennington: technician responsible for preparation of morphological and neurochemical tissues, digitization, analysis.
Mr. H. Cheng: technician responsible for biochemical assays
Mr. K. Zanewich: BSc., BEd., part-time computer programmer
Dr. D. Hoar, Associate Professor, Co-investigator

Students: S. Faust
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COLLABORATORS

Dr. Ken Yoshida, Industrial Hygienist, Dept of Community Health, University of Calgary
Dr. M.G. Prior, Alberta Environmental Centre, Vegreville,
Dr. P.N. Nation, Head of the Pathology Branch, Animal Health Division, Alberta Agriculture Center
Dr. F. Green, Associate Professor, Depatment of Pathology, University of Calgary

PUBLICATIONS

Hannah, R.S., Hayden, L.J. and Roth, S.H. Hydrogen sulfide exposure alters the amino acid content in developing rat CNS. Neurosci. Lett. 99: 323-327. 1989.

Hayden, L.J., Franklin, K.J., Roth, S.H. and Moore, G.J. Inhibition of oxytocin-induced but not angiotensin-reduced rat uterine contractions following exposure to sodium sulfide. Life Science 45: 2557-2560.

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Hayden, L.J. Pui, A.C. and Roth, S.H. Human lung fibroblast cytotoxicity following acute sodium sulfide exposure. Proc. West Pharmacol. Soc. 1990. (Accepted for publication)

Hayden, L.J., Goeden, H. and Roth, S.H. Normal growth and development in the rat during sub-chronic exposure to low levels of hydrogen sulfide. Submitted to Journal of Toxicology and Industrial Health.

Hayden, L.J., Goeden, H. and Roth, S.H. Exposure to low levels of hydrogen sulfide elevates circulating glucose in maternal rats. Submitted to Journal of Toxicology and Environmental Health.

Hannah, R.S. and Roth, S.H. Hydrogen sulfide exposure and Purkinje cell growth patterns in the developing rat cerebellum. Submitted to Brain Res.

Roth, S.H. and Mathison, H. Complex actions of hydrogen sulfide and its volatile salts on the discharge activity of the crayfish sensory neuron. To be submitted to Neurotoxicology.

Manuscripts in Preparation

Roth, S.H. and Wright, J.D. The application of hydrogen sulfide alone and in the presence of acetylcholini on an isolated sensory neuron.

Pui, A.C., Hayden, L.J. and Roth, S.H. Cytotoxicity of sodium sulfate on W1-3I human lung fibroblast cells.

Hayden, L.J., Wetmore, D., Roth, S.H. and Goeden, H. Irreversible inhibition of tissue cytochrome oxidase following sub-chronic exposure to low doses of hydrogen sulfide.

ABSTRACTS

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R.S. Hannah, R. Benningtonand S.H. Roth. A relationship between hydrogen sulfide exposure and taurine levels in maternal rats. In press.

PRESENTATIONS

June 1986, Alberta Environmental Research Centre, Vegreville, Alberta

November 1986, Research Interest Group,Vocational and Rehabilitation Research Institute, Calgary "Behavioural Toxicology"

February 1987, American Socity of Toxicology, Washington

April 1987, 3rd International Symposium of the Molecular Basis of the Action of Drugs and Toxic Substances, California.

October 1988,Endocrine Research Group, University of Calgary "Low dose hydrogen sulphide toxicity in vitro"

March 1989, University of Saskachewan,Toxicology Research Centre "Toxicity of hydrogen sulphide"

March 1989, Endocrine Research Group, University of Calgary, "Sodium sulphide and receptor function"

June 1989, International Symposium on Hydrogen Sulphide Toxicity, "Hydrogen sulphide in biochemical systems"
"The neurophysiological effects of low concentrations of hydrogen sulphide on the developing and mature nervous systems"

October 1989, University of Western Ontario,"The effects of low dose hydrogen sulphide exposure on dendritic arborization in the rat cerebellum"

November 1989, McMaster University,"Hydrogen sulphide and the developing brain"

RECOMMENDATIONS

The most important recommendation is to continue the study of H_2S toxicity, to elucidate its mechanism of action and to provide data which can be useful in the clinical situation, either for treatment of exposure or for prevention of adverse effects on the developing system. It is recognized that H_2S does not exist in the environment in isolation, thus for a more realistic study the effect of combinations must be examined. This would involve other environmental agents as well as ethanol, drugs, etc.The most severe criticism of the study would be the number of test subjects that may have not been large enough of a population to identify significant "subtle" effects. This is one of the major reasons to develop in vitro assays that can be easily conducted with large populations.

Continued research is dependent on securing sufficient funding, and this has become a major problem for all the laboratories. Until this problem is solved, the research is not likely to continue and the expertise will find other projects.

EVALUATION OF THE STUDY

This research project began just three years ago. The experiments were designed to determine whether low levels of H_2S are potentially toxic. We exmained a rather broad screen of parameters that would provide data for both a systematic analysis of the toxicological effects of H_2S, as well as a basis for direction of future studies and development of screening techniques, mechanistic explanations and improved therapeutic regimens in cases of exposure or "knockdown". We did establish that exposure to low levels of H_2S does produce toxic effects on the devloping organism. These were often subtle, but significant and are likely to result in behavioral deficits.This study, therefore provides support for the existence of an **"H_2S Syndrome"**. Athough there is not an adequate denition or diagnostic measure of this syndrome, we must not ignore the potentially severe changes that could occur to the developing organism upon exposure. The effects observed in this study were similar to those we and others have reported with CNS-active drugs such as barbiturates, phenothiazines and benzodiazepines, and thus are very suggestive that H_2S has the potential of being a behavioral teratogen to humans. Our results are not absolutely conclusive and must be tested further using different techniques and approaches. It is recognized that the original objectives represented a major research program that would require many man-years of investigation. Research involving inhalation exposure paradigms , multidiscipline evaluation and developmental studies are very tedious and labour intensive. The project progressed very successfully, but clearly demonstrates that continued efforts are required, for example chronic ,i.e. 90 day and longer studies , as well as examination at very low concentrations.

Considerable data has been generated, the majority of objectives were attempted. It was somewhat ambitious to expect that all the objectives would have been successfully completed in the time frame allowed. As expected the research generated many new problems, but also provided guidance for focused research. Many of the results highlighted the "subtle" nature of the toxicity of H_2S at low levels, and also the

dominant effects on the developing nervous system. The large volume of negative effects is the most difficult to justify , but the lack of effects can now be documented with confidence.

DESCRIPTION OF THE PROJECT

It is well recognized that hydrogen sulphide is extremely toxic to animals and humans at high conenctrations. The National Institute for Occupational Safety and Health (NIOSH) of the U.S. reported in the Criteria for a Recommended Standard Occupational Exposure to Hydrogen Sulphide (publication no.77-158, 1977) that hydrogen sulphide was a leading cause of sudden death in the workplace. Hydrogen sulphide is a major component of sour gas and is also the by-product of more than 70 industrial operations. A recent report of the Alberta Health (Report on H_2S Toxicity 1988), presented an excellent and complete review of the effects of H_2S including both animal and human experimental data. It was stated that "H_2S by itself is a broad spectrum toxicant that can elicit numerous psychological and biological responses....all organ systems respond variably to different levels of H_2S with no given level affecting all systems equally at the same time or rate". It was concluded "there remains a lack of good scientific data about the long-term, low level chronic effects..." and identified that "certain factors may increase susceptibility of humans to H_2S" i.e. "age, as in the case of the very young".

Hydrogen sulphide was selected as a prototype environmental toxicant because of its widespread occurence. The Workman's Compensation Board of Alberta has dealt with over 40 claims per annum resulting from "knockdown" by H_2S, and in 1988 four deaths were attributed to H_2S. Although most of the exposures are related to the occupational environment such as the oil and gas industry, severe risks occur to the general population at large.

The present study proposed a multi-disciplinary examination of the effects of acute and chronic low levels of H_2S on the <u>developing</u> mammalian system using the rat as a model. Additional parallel studies were to examine the mechanisms of H_2S toxicity using <u>in vitro</u> models and cultured cell systems. The original experiments were designed to provide a rather broad screen of parameters that would provide data for both a systematic analysis of the toxicological

effects of H_2S as well as a basis for direction of future studies and development of screening techniques, mechanistic explanations and improved therapeutic regimens.

Hydrogen sulphide can produce a variety of psychological and neurophysiological reactions, for example, nervousness, headache, lightheadedness, sleep disturbances, insomnia, drowsiness, fatigue, weakness of extremities, spasms, disturbed equilibrium, vertigo, convulsions, anxiety, agitation and delirium. Exposure to high concentrations can produce severe symptoms such as deep coma, nerve paralysis, unconsciousness, paralysis of the respiratory centre, cardiac failure, and death.A number of symptoms upon recovery have also been described including many of the above as well as lack of initiative, irritability, anxiety, poor memory, decreased libido, and nystagmus. Residents living in the surrounding environment of Lodgepole, Alberta which experienced a blowout in 1982, complained of headache, tiredness, numbness, emotional and visual disturbances and loss of smell. Depression of the nervous system can occur at 200 ppm, therefore, it is most likely that the systemic effects on the nervous system could result in paralysis of the respiratory center leading to death. This strongly suggests the central nervous system is a major target organ for H_2S toxicity.

It is recognized that the original objectives represented a major research program that would require many man years of investigation. Research involving inhalation exposure paradigms, multidiscipline evaluation and developmental studies are tedious, prolonged and comparatively slow to completion. The project progressed very successfully and demonstrated that continued efforts, i.e. chronic 90 day exposures are necessary before any definitive statements can be made regarding H_2S toxicity on the <u>developing and maturing</u> organism.

The overall objective of the study was to determine whether low levels of hydrogen sulphide (H_2S) can affect various tissues of the mature and immature (developing) mammalian system using the rat as the test animal.

The project was designed as a multidisciplinary project in order to include behavioral, morphological, neurological, biochemical and genetic studies. We focused on the developing system because of the potentially greater susceptibility of this age group. The majority of the objectives were completed, and in the end we realized that the study created more questions. Many of the findings were found to be not significant.These are difficult to justify in a progress report, however it was absolutely necessary that these data be obtained in a well controlled study, and now the results can be documented with confidence. During the initial phase of the project we designed and fabricated an ideal exposure system for conducting whole animal exposures that is now being developed for commercial distribution by a company in Calgary. The techniques utilized in this study were constantly refined and resulted in routine procedures that were consistent and reliable.

The development of the rat fetus and neonate appears to be normal with no evidence of any overt teratological effects on exposure to low levels of hydrogen sulphide. We also conclude that routine parameters indicative of normal development are not significantly different in the exposed group (20, 50 and 75 ppm H_2S) from the control group.There did not appear to be any significant differences in hematological parameters such as hematocrit, nor pathological or histopathological parameters between exposed or control groups.

A number of significant effects, however, did occur. We observed prolongation of parturition or delivery termed dystocia in approximately 18% of the 75 ppm exposed group.In vitro studies provided evidence that this phenomenon may be a result of changes in the receptors that bind oxytocin which is involved in muscle contraction during labour. Serum glucose levels of exposed dams were elevated and this was accompanied by a dose-dependent decrease in serum triglycerides and elevation of serum cholesterol. Further studies are required to establish whether these effects are meaningful and to provide any explanations. Overall we did not find any major alterations in the biochemical profiles of either the dams or the pups

exposed to low levels of H_2S, but there were several indications that changes in some metabolites and brain enzyme activity may be altered for short periods of time. Initial studies revealed that the liver cells of exposed animals may be genetically altered but these effects are not conclusive. Preliminary studies using cells in culture also demonstrated that cell growth could be inhibited following a single exposure to hydrogen sulphide.

Our results indicate that the major effects of H_2S appears to occur in the developing central nervous system. We have documented that H_2S at the concentrations studied changes levels of chemical transmitters in the developing rat brain during the critical phase of development, and that the growth characteristics and morphology of specific neurons may be altered.

Many of the results emphasize the "subtle" nature of the toxicity of low levels of hydrogen sulphide. We may not have identified threshold effects because of the concentration range examined, but it is very suggestive that these may be considerably lower than previous estimates.

The project still exists in the "beginning stages", but continued research is dependent on sufficient funding. Until a solution to this problem is found, many of the questions concerning hydrogen sulphide toxicity will remain unanswered, and the findings of the present study will not be utilized to the fullest extent.